Metabolism of Nemacur [Ethyl 4-(Methylthio)-*m*-tolyl Isopropylphosphoramidate] and Identification of Two Metabolites in Plants

T. Bill Waggoner

Nemacur-^{14}C,^{3}H was injected into the stems of beans, tomatoes, peanuts, and potatoes and the plants were harvested at 7, 14, 21, and 28 days. Two major metabolites were identified by tlc, isotopic ratios, ir, and ms as II and III. Two minor metabolites, IV and V, consisting of intact phosphoramidate structures, never accounted for more than 1.5 and 0.2%, respectively, of the total residue. Their exact structures were not determined. The significant acetylcholinesterase inhibiting residues consisted of I, II, and III, and the remainder of the radioactivity consisted of breakdown products. Compounds II–IV were more active as acetylcholinesterase inhibitors than I. The phenols from II and III were detected in the organosoluble fraction. An analytical residue method for plants should include I–III.

The purpose of this study was to identify the major metabolites of ethyl 4-(methylthio)-*m*-tolyl isopropylphosphoramidate (I) in plants (Figure 1). The oxidation of the CH_3S group was expected, since earlier work showed that it was a precursor to CH_3SO and CH_3SO_2 in biological systems (Benjamini *et al.*, 1959). The presence of these oxidation products would need to be incorporated in the development of an analytical residue method for plants (Thornton, 1971).

EXPERIMENTAL

Labeling of I. Separate dual-labeled experiments were conducted utilizing carbon-14 (^{14}C) and tritium (^{3}H): Experiments 1–6, $^{14}C_1$-ethyl 4-(^{3}H-methylthio)-*m*-tolyl isopropylphosphoramidate, ^{3}H dpm/^{14}C dpm ($^{3}H/^{14}C$)—2.5; and Experiments 7–12, ethyl 4-(^{3}H-methylthio)-*m*-tolyl $^{14}C_2$-isopropylphosphoramidate, $^{3}H/^{14}C$—2.1. Specific activities were $^{14}C_1$-ethyl—1.62 mCi/mmol, $^{14}C_2$-isopropyl—0.23 mCi/mmol, and ^{3}H-methylthio—12.9 mCi/mmol.

Standards. Unlabeled I was used for the preparation of the corresponding sulfoxide (II) and sulfone (III) by oxidation with H_2O_2 and $KMnO_4$ to give II and III, respectively (Figure 1).

Plants. Top Crop bush beans (*Phaseolus vulgaris*), Rutgers tomatoes (*Lycopersicon esculentum*), and Mammoth Jumbo Peanuts (*Arachis hypogaea*) were started indoors in Jiffy-7 peat pellets under fluorescent lights. The dry compressed peat pellets (0.5-in. height) expanded to a 4-in. height when soaked in water for 30 min. The seeds were germinated in the expanded pellets and the seedlings were transferred to individual clay pots (one plant per pot) and planted in sand while retaining the peat pellets intact. Five days prior to treatment with I, the plants were moved outdoors. The ages at the time of treatment were 4 weeks (beans) and 6 weeks (tomatoes and peanuts).

Kennebec white potatoes (*Solanum tuberosum*) were started and grown indoors in 6-in. clay pots. Six potato plants per pot were treated at age 6 weeks.

Treatment. Each plant was injected in the main stem with 20–50 μl of an ethanol:water (1:1) solution of I at a position 1–2 in. above the soil level. Treated plants were harvested at 0, 7, 14, and 28 days (three plants per interval). Zero-day intervals were 2–3 hr after treatment.

Thin-Layer Chromatography (tlc). Fresh precoated glass plates (Brinkmann) of silica gel F_{254} were stored in a desiccator and used without further activation. Three solvent systems were utilized (v/v): Solvent 1, chloroform:ethanol (24:1), Solvent 2, ether:acetone (9:1), and Solvent 3, ether:acetone (1:1).

Extraction Procedure. The main stem was cut at the soil level and the entire plant was chopped with dry ice in a blender. The sample was then stored in the freezer while the dry ice sublimed within 24 hr.

Approximately 20 g of the fresh homogenous plant material were mixed with 20 g of sodium sulfate in a blender for 2 min with 200 ml of acetone. The mixture was filtered and the filter cake was reextracted with an additional 100 ml of acetone. The extracts were combined and concentrated *in vacuo* to 25 ml. Aliquots of the concentrated acetone extract were fractioned by tlc with ether:acetone (1:1) and the areas of radioactivity were eluted with acetone or methanol. The fractions were each concentrated *in vacuo* at 30° C and rechromatographed with chloroform:ethanol 24:1). The extracted plant solids were analyzed for radioactivity.

Purification of Metabolites. A portion of the crude acetone extract was chromatographed on silica gel preparative thin-layer plates (8 × 8 in.) with Standards. The radioactive areas corresponding to the respective standards were eluted with acetone. The eluant was concentrated and rechromatographed using solvent systems 1, 2, and 3 to give a single radioactive component free of impurities.

Infrared (ir) Spectra. After purification of the metabolite, the compound (approximately 20 μg) was concentrated on the tip of a KBr Wick-Stick (The Harshaw Chemical Co.) using acetone as the developing solvent. A 0.5 mm pellet was

Chemagro, a Division of Baychem Corporation, P.O. Box 4913, Kansas City, Missouri 64120.

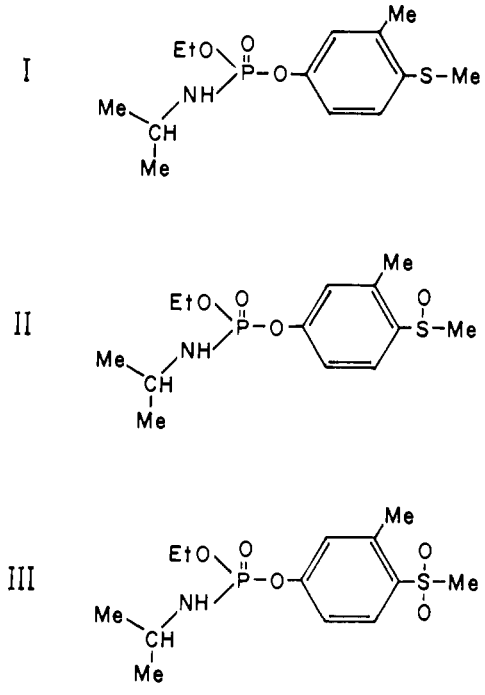

Figure 1. Structures of ethyl 4-(methylthio)-*m*-tolyl isopropylphosphoramidate (I), ethyl 4-(methylsulfinyl)-*m*-tolyl isopropylphosphoramidate (II), and ethyl 4-(methylsulfonyl)-*m*-tolyl isopropylphosphoramidate (III)

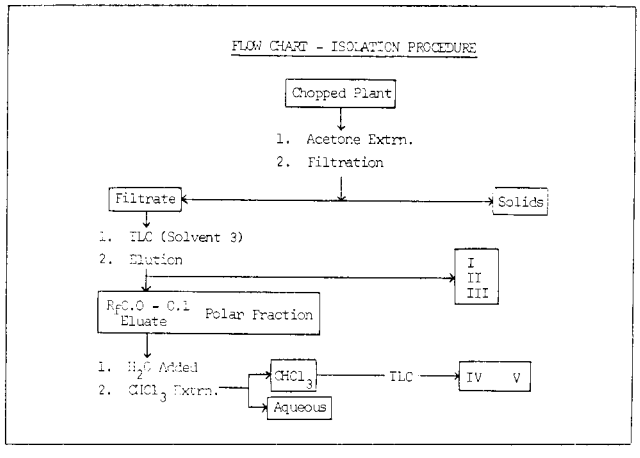

Figure 2. Flow chart for isolation of metabolites

prepared from the tip of the Wick-Stick with an Ultra Micro Die (No. 186-007, Perkin-Elmer Corp.). The actual amount of compound in the micropellet was 5–10 μg. The spectra were determined on a Perkin-Elmer Model 21 recording spectrophotometer equipped with a 6× microsampling unit, beam condenser (Perkin-Elmer, No. 196-0011). Standards (20 μg) were also carried through the purification procedure starting at the tlc stage prior to the preparation of their KBr pellets.

Mass (ms) Spectra. The metabolites and standards were purified as above. The compounds were then concentrated into capillaries (5 μl capacity) suitable for use in the direct probe. Spectra were determined on a quadrupole laboratory mass spectrometer (Finnigan Instruments, Model 1015).

Counting Procedure. Radioactivity was determined by a modified wet combustion technique consisting of perchloric acid–hydrogen peroxide digestion of samples, solubilization, and subsequent liquid scintillation counting (Mahin and

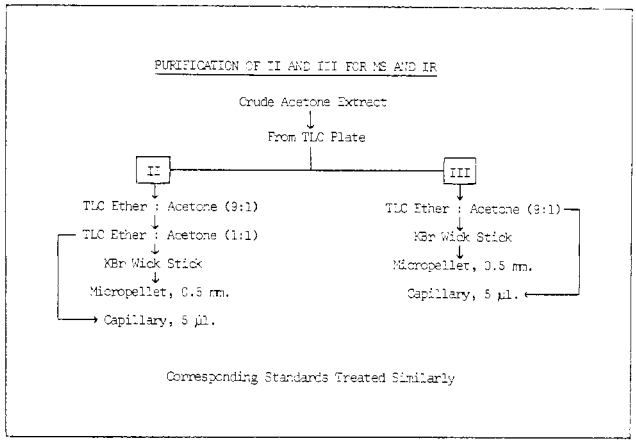

Figure 3. Purification of metabolites for ir and ms analysis

Lofberg, 1966). A 0.2 g (or 0.2 ml) sample was weighed (or pipetted) in a counting vial. Hydrogen peroxide (0.2 ml of 30%) and perchloric acid (0.2 ml of 70%) were added. The vial was stoppered with a cap lined with a silicone gasket and heated to 75° C in an oven until the contents were colorless (30–45 min). The vial was cooled in Dry Ice–acetone. Concentrated ammonium hydroxide (0.2 ml) was added to the vial followed by 8.0 ml of ethyleneglycol monomethyl ether and finally 10 ml of Spectrafluor (Nuclear Chicago, 168 ml diluted to 1 l. with toluene).

Samples were counted with a Packard Model 3375 liquid scintillation spectrometer. Tlc plates, 2 × 8 in., were scanned with a Packard Model 7201 radiochromatogram scanner equipped with a tlc scanning attachment. Plates, 8 × 8 in., were scanned with a Varian Aerograph Model 6000-10 thin-layer scanner.

Acetylcholinesterase Assay. An *in vitro* system consisted of 20 mg of acetylcholine bromide (Sigma Chemical Co.), 18.6 mg of $MgCl_2$, 30 mg of NaCl, 0.2 mg of acetylcholinesterase, Type IV from horse serum (Sigma Chemical Co.), 0.025–0.050 ml of Standards or IV, and enough 0.01 *N* NaOH to give a final volume of 5.5 ml and pH 8.0. Standard or IV was incubated with the enzyme for 1 hr before the reaction was initiated by addition of the substrate. The rate was determined by following the pH change, initially at 8.0 during the first 2.5 min of reaction time. Readings were observed at 30-sec intervals. The formation of $[H^+]$ was proportional to the enzyme concentration in this system and was linear from 0 to 2.5 min reaction time at a constant rate. The amount of I, II, III, and IV required to decrease the rate of reaction by 50% (I_{50}) was determined.

RESULTS AND DISCUSSION

The general isolation scheme for all fractions is shown in Figure 2. Tentative identifications of I, II, and III were made from tlc data and $^3H/^{14}C$ ratios. The relative mobilities for all tlc systems were I > III > II. In Experiments 1–6, the isotopic ratios of isolated I, II, and III were 2.5, which was identical to the ratio of I administered. In Experiments 7–12, the $^3H/^{14}C$ ratios of isolated I, II, and III were 2.1, which was identical to the ratio of I administered. Therefore, the phosphoramidate structures were assumed to be intact.

The distribution of radioactivity for Experiments 1–12 is summarized in Table I. The polar fraction represented the radioactivity from the area R_f 0.0–0.1 on the silica gel plate.

At intervals 1–3 days posttreatment, approximately 5%

Table I. Distribution of Radioactivity In Plants From Nemacur-^{14}C

Expt no.	Interval, days	Plant	% Distribution I	II	III	Polar fraction	Solids	% total recovery
$^{14}C_1$-Ethyl								
1	0	Tomato	38	48	0	11	3	93
2	7	Tomato	0	33	33	22	12	38
3	7	Peanut	2	35	15	15	33	47
4	14	Peanut	0	15	10	15	60	25
5	14	Bean	0	31	0	7	62	24
6	28	Bean	0	5	0	32	63	9
$^{14}C_2$-Isopropyl								
7	0	Tomato	38	48	0	10	4	100
8	7	Tomato	0	41	31	16	12	24
9	7	Peanut	10	48	8	10	24	21
10	14	Peanut	3	36	6	12	43	45
11	14	Bean	0	33	0	38	29	35
12	28	Bean	0	21	0	66	13	26

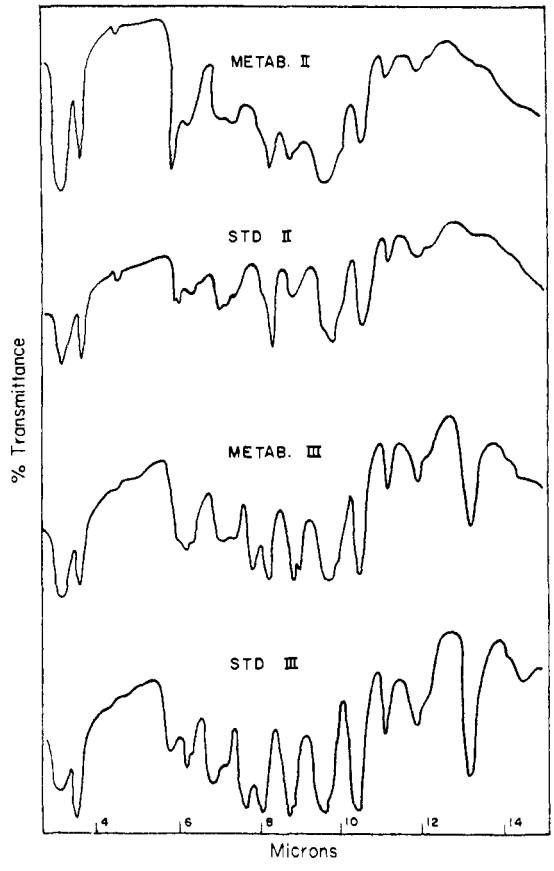

Figure 4. Ir spectra of metabolites II and III and their corresponding standards

of the radioactivity in the acetone extract moved with the solvent front on thin-layer plates using solvent 3 (Figure 2), and contained only carbon-14. Subsequent tlc on silica gel plates developed with *n*-hexane indicated at least four compounds which were probably naturally occurring, but were not identified in this study.

Since the highest concentration of II and III occurred about 7 days after treatment (Table I), additional amounts of metabolites were isolated at this interval to confirm the structures. Six tomato plants were injected with $^{3}H/^{14}C$-ethyl parent plus 1 mg of unlabeled I. Some phytotoxicity occurred at the injection site. Compound I and metabolites II and III were isolated at the 7-day interval for subsequent confirmation of structure. The purification and preparation for ir and ms analyses are summarized in Figure 3.

The ir spectra of the metabolites and their corresponding standards are shown in Figure 4. Absorption from 5.6–5.8 μ was due to residual acetone in the micropellet. After ir analysis, the micropellets were extracted. The actual amounts in the micropellets were calculated from the radioactivity recovered in the extract. The $^{3}H/^{14}C$ ratio was unchanged.

The molecular ion peaks of the metabolites from ms analysis were 319 for II and 335 for III. Identical molecular ion peaks was observed with the corresponding standards. A molecular ion peak of 303 was observed for Standard I.

Small amounts of other metabolites, Compounds IV and V, were partitioned into chloroform from the aqueous polar fraction (Figure 1). For Experiments 1–12, Compound IV never accounted for more than 1.5% of the total residue in the plant and was absent at 28 days posttreatment. Compound V never accounted for more than 0.2% of the total residue in the plant and was absent at 7, 14, and 28 days posttreatment. The structures of IV and V were not confirmed, but their $^{3}H/^{14}C$ ratios corresponded to that of I, indicating intact phosphoramidate structures.

For isolation of IV, six potato plants were stem-injected with $^{14}C_1$-ethyl 4-(methylthio)-*m*-tolyl isopropylphosphoramidate diluted with 50 mg of unlabeled I. The plants were harvested and extracted with acetone 40 hr posttreatment. The extract was concentrated *in vacuo* at 30° C to a thick syrup. Water (50 ml) was added and the aqueous solution was extracted three times with 50 ml of chloroform. The combined chloroform extracts contained 97% of the total radioactivity solubilized. Compound IV was isolated on silica gel preparative plates developed with acetone and comprised 7.4% of the total residue in the chloroform extract.

Compound IV (R_f 0.35) was more polar than standard II (R_f 0.64) for the silica gel–acetone system. Attempts to identify a molecular ion peak of IV from ms analysis were unsuccessful. Basic hydrolysis of IV gave a product which showed an R_f identical to the corresponding sulfoxide phenol on tlc. The molecular ion peak of the unknown was 170 from ms analysis, the same as observed for a sulfoxide phenol standard. Further investigations of IV and V are being continued.

Table II. Inhibition of Acetylcholinesterase by Nemacur and Its Metabolites

Compound	I_{50} Molar concentration	I_{50} pg/mg enzyme	Relative inhibition % of I
I	18.0×10^{-9}	200	100
II	9.7×10^{-9}	120	167
III	9.7×10^{-9}	120	167
IV (unknown)	3.6×10^{-9}	30	670

The relative inhibition of acetylcholinesterase by I and its metabolites is shown in Table II. The concentrations of IV were based on mol wt 303. The enzyme system was based on that described by Nachmansohn and Wilson (1955).

The greater activity of II and III was consistent with earlier findings, where CH_3SO and CH_2SO_2 in the para position of 4-substituted 2,5-xylenyl diethyl phosphates showed increasing I_{50} values over CH_3S as inhibitors of housefly-head cholinesterase (Metcalf *et al.*, 1964).

The I_{50} values for I–IV fall within the range of the majority of organophosphates (O'Brien, 1960). The values for I–III were from standards, whereas the value for IV was from the isolated compound.

The increased activity of IV could be a result of the oxidation of the amido portion shown previously for schradan (O'Brien, 1960). A loss of the isopropyl substituent to give an unsubstituted amidic acid was not expected, based on the mechanism of hydrolysis of phosphoramidates (Garrison and Boozer, 1968).

The chloroform extracts from the polar fraction (Figure 2) consisted of two compounds containing only tritium. These were identified as the free phenols of II and III by tlc. Hydrolysis of phosphoramidates in biological systems has been shown to be a major pathway (St. John and Lisk, 1970).

The distribution of radioactivity ($^3H/^{14}C$—2.6) in the plant solids from $^{14}C_1$-ethyl label was highest at 28 days posttreatment comprising 63% of the total residue. The solids were reextracted with ethanol:water (16:1) in the blender and an additional 17% was solubilized. The extract contained mostly tritium and the $^3H/^{14}C$ ratio in the solids decreased to 1.1. The radioactivity in the solids was attributed to breakdown of the phosphoramidate structure, and therefore the unextractable residue did not contribute to the total toxic residue.

The results of this study show that residues of Nemacur in plants consist mostly of the sulfoxide (II), smaller amounts of sulfone (III), and very little if any sulfide (I) at intervals of 7 days and greater. Therefore, an analytical residue method must include I–III for determining the total cholinesterase inhibiting compounds. The low amounts of IV and V which were found do not contribute significantly to the toxic residues. The study of acetylcholinesterase shows that the metabolites are more active than I; however, the relative toxicities of I–IV to mammalian organisms are unknown.

LITERATURE CITED

Benjamini, E., Metcalf, R. L., Fukuto, T. R., *J. Econ. Entomol.* **52,** 94 (1959).
Garrison, A. W., Boozer, C. E., *J. Amer. Chem. Soc.* **90,** 3486 (1968).
Mahin, D., Lofberg, R., *Anal. Biochem.* **16,** 500 (1966).
Metcalf, R. L., Fukuto, T. R., Frederickson, M., J. AGR. FOOD CHEM. **12,** 231 (1964).
Nachmansohn, D., Wilson, I. B., in "Methods in Enzymology," S. P. Colwick, N. O. Kaplan, Eds., Vol. I, Academic Press Inc., New York, N.Y., 1955, pp 642–651.
O'Brien, R. D., "Toxic Phosphorus Esters," Academic Press Inc., New York, N.Y., 1960, pp 65–67, 73.
St. John, Jr., L. E., Lisk, D. J., J. AGR. FOOD CHEM. **18,** 125 (1970).
Thornton, J. S., J. AGR. FOOD CHEM. **19,** 890 (1971).

Received for review June 7, 1971. Accepted August 18, 1971. Presented in part at the Division of Pesticides Chemistry, 158th ACS National Meeting, New York, N.Y., September 8–12, 1969.